Retinoic Acid Receptor Gamma Mediates Topical Retinoid Efficacy and Irritation in Animal Models

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Among retinoic acid receptors (RARs) α, β, and γ, the messenger RNA level of RAR-γ is the most readily detectable by Northern blotting in human and mouse skin. This observation suggests that RAR-γ may play a critical role in the modulation of the therapeutic benefits and side effects of retinoids in skin. To test this hypothesis, 11 RAR-γ selective retinoids were synthesized based on three related structures. Each compound was found to prefer RAR-γ when assessed by retinoid-induced transcriptional activity (RAR-γ > RAR-β > RAR-α). The apparent K_d for binding to recombinant receptor protein was found to follow a similar trend. To correlate this receptor selectivity with *in vivo* activity, the compounds were tested topically in the Rhino mouse utriculi reduction and rabbit irritation models, two assays widely used to screen retinoids for efficacy and side effects, respectively. The results indicated that for these compounds, both efficacy in the utriculi reduction assay and irritation potential in rabbits correlated positively with the RAR-γ transactivation activity, with r^2 of 0.9 and 0.8, respectively. These data suggest that RAR-γ is an important regulator of retinoic acid efficacy in skin and further, that the irritation associated with the use of retinoids is most likely a receptor-mediated process. ***Key word: Rhino mouse. J Invest Dermatol 104:779–783, 1995***

The recent discovery of three different retinoic acid receptors (RAR-α, -β, and -γ) [1–5] raises the questions of whether each receptor plays a distinct biologic role and, if so, whether the retinoid efficacy [6–10] and side effects [11,12] can be separated. To address these questions, receptor-selective or specific retinoids are needed. Despite the high level of homology among the three RAR receptors, RAR-γ–selective retinoids have been reported [13,14].

One of the most commonly used animal models for evaluation of the *in vivo* efficacy of retinoids is the Rhino mouse utriculi reduction assay. The Rhino mouse is a strain of the hairless mouse that possesses large comedones resembling those seen in acne in humans [15,16]. The comedones decrease in size in a dose-dependent manner with the addition of retinoic acid, and this decrease is taken as predictive of retinoid efficacy in acne. Yet the mechanism by which retinoic acid mediates this response is unknown.

Another commonly used *in vivo* system for evaluation of retinoid activity is the rabbit irritation model [17]. This model monitors side effects such as erythema, edema, and scaling, commonly associated with retinoid treatment [11,12]. The molecular basis of retinoid-induced irritation has long been a matter of speculation because no single biochemical marker defines this process.

The efficacy and side effects found in the clinical use of retinoids and assessed using these animal models may be mediated through different receptors. Synthetic retinoids with preferential affinity for a given receptor would be useful for defining the roles of each receptor in these animal models. In addition, it may be possible to optimize efficacy while reducing unwanted side effects in the design of future retinoid analogs. To test this hypothesis, we synthesized and characterized 11 retinoids derived from three known chemotypes [13,14,18] as RAR-γ selective. A close correlation was found between RAR-γ–mediated transcriptional activity, receptor binding, and efficacy in both the Rhino model and the rabbit irritation model. These results suggest that RAR-γ mediates important retinoid-induced effects in the skin, as might be predicted by the tissue distribution of this receptor [19], and that retinoid-induced skin irritation is most likely a receptor-mediated event.

MATERIALS AND METHODS

Animals Male New Zealand White rabbits, 10–12 weeks old, were purchased from the MVR farm (Loudonville, OH). Pathogen-free 9–12-week-old female hairless Rhino mice (hr^{rh}/hr^{rh}) were bred by Taconic Laboratories (Germantown, NY). Animals were kept on a 12-hour light/dark cycle. The animal rooms were supplied with yellow lights to avoid retinoid photoisomerization. All experiments were performed in accordance with National Institutes of Health guidelines for animal handling.

Chemicals All chemicals used were of reagent grade and obtained from Sigma Chemical Co. (St. Louis, MO). All-trans retinoic acid (t-RA) was purchased from BASF (Wyandotte, MI). Compounds 1, 7, and 11 were prepared as reported previously [13,14,18]. All other synthetic retinoids were synthesized at BMS Central Chemistry (Wallingford, CT).

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Abbreviation: t-RA, all-trans retinoic acid.

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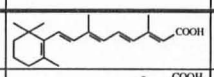
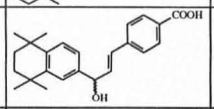
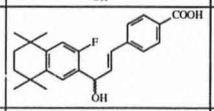
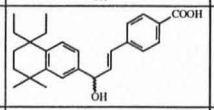
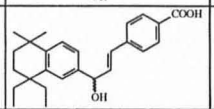
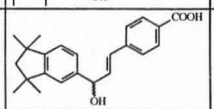
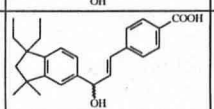
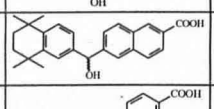
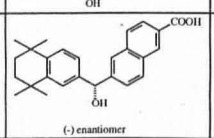
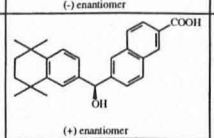
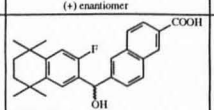
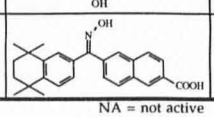
#	Structure	Transactivation EC50 (nM)			Direct Binding Apparent Kd (nM)			Rhino ED30	Rabbit IS3
		α	β	γ	α	β	γ	(mM)	(mM)
tRA		31	8	7	0.9	0.4	0.2	0.015	0.3
1		442	271	20	1900	1400	60	0.065	0.23
2		2200	550	48	2000	2000	350	0.21	2.5
3		NA	500	40	1100	1100	75	0.16	ND
4		500	350	70	400	250	275	0.17	ND
5		NA	NA	100	1500	1400	250	0.27	2.4
6		NA	600	60	2000	1400	125	0.12	ND
7		NA	575	163	NA	654	87	0.22	0.98
8	 (-) enantiomer	NA	363	58	NA	491	97	0.14	0.98
9	 (+) enantiomer	NA	NA	450	NA	4065	1122	0.75	74
10		NA	NA	300	NA	1350	400	0.58	29
11		NA	450	30	900	50	5	0.072	0.037

NA = not active ND = not determined

Figure 1. *In vitro* and *in vivo* activity of RAR-γ–selective retinoids. The RAR-γ selectivity of the 11 retinoids was demonstrated in a transient transactivation chloramphenicol acetyltransferase assay in HeLa cells and by binding to recombinant receptor protein. *In vivo* efficacy and irritation were tested in the Rhino mouse utriculi reduction (n = 5) and the rabbit repeat irritation (n = 6) experiments, respectively.

***In Vitro* Assays** Retinoid selectivity was assessed using a transactivation assay and a direct binding assay. In the transactivation assay, HeLa cells were transfected with a Gal4-RAR (DEF) α, β, or γ chimeric receptor, along with a reporter plasmid containing the Gal4 response element and a chloramphenicol acetyltransferase reporter gene. β-galactosidase was included as a control for transfection efficiency. Transfected cells were grown in the presence of the test retinoid for 24 h, then harvested and assayed for chloramphenicol acetyltransferase concentration using an enzyme-linked immunosorbent assay (5′ to 3′, Inc., Philadelphia, PA). In each experiment, a t-RA dose-response was included as the positive control. The EC_{50} listed in **Fig 1** is the concentration of the test material reaching 50% of t-RA maximum stimulation at 10^{-6} M. In the case of RAR-α of compound 2, the transactivation activity at 10^{-6} M was 45% of t-RA maximum, and the data were extrapolated slightly above 10^{-6} M to give the estimated K_d of 2.2 μM. Compounds with a transactivation activity well below 50% of t-RA activity at 10^{-6} M are listed as "not active" in **Fig 1**.

For the binding assay, recombinant RAR protein was expressed in *Escherichia coli*, and the apparent dissociation constant was determined by the charcoal absorption method [20]. Briefly, serial dilutions of the test retinoids (10^{-11} to 10^{-5} M) were made in dimethylsulfoxide in a volume of 100 μL. Twelve micrograms of crude cytosolic extract prepared from pET15b/hRAR-α, -β, or -γ prepared protein was used for each data point. All reactions were carried out in binding buffer (60 mM NaImidazole, 500 mM NaCl, 20 mM Tris, pH 7.9) for 14–16 h at 4°C in a final volume of 1 mL. Unbound [^{3}H]-RA was removed by addition of 0.5 mL of equivalent-sized dextran-treated charcoal (final concentration 3% [wt/vol]) for 15 min at 4°C [21]. One half milliliter of the supernatant was removed, added to 12 mL of ReadySafeTM scintillation cocktail (Beckman), and counted in an LS6500 scintillation counter (Beckman).

***In Vivo* Assays** Retinoids in 50 μl ethanol were applied topically to the central dorsum of Rhino mice [16] once daily for 5 d. Seventy-two hours after the last treatment, the animals were sacrificed by CO_2 inhalation. A full-thickness skin biopsy specimen (7/8 in.) was removed from the treatment site. The epidermis was separated from the dermis by overnight incubation in 0.5% acetic acid at 4°C. Epidermal sheets were fixed in 10% formalin, dehydrated with ethanol, and cleared in xylene. The diameter of 40 utriculi was measured for each sample of processed epidermis with an image analyzer (Image Measure; Microscience, Federal Way, WA). For each retinoid, a dose ranging from 3.3 μM to 3.3 mM was tested. The percent utriculi reduction was defined as the percent decrease of utriculi diameter compared with that of the ethanol control. The ED_{30} value (dose rendering 30% reduction of utriculi diameter, i.e., half-maximal biologic response) was calculated by interpolation of the dose-response curve using linear regression analysis. Five animals were used in each group.

The irritation potential for each of the test compounds was assessed using the 14-day repeat rabbit irritation assay [17]. Each rabbit was clipped at four dorsal sites (16 cm^2 for each site). Each site was randomized for treatment with a retinoid at three concentrations, and t-RA at 0.33 mM was used as a positive control. Each retinoid was applied topically in ethanol (200 μL) once daily for 14 consecutive days. Each day before administering the compound, the degree of erythema, edema, and scaling was assessed visually using the Draize 0–4 grading scale [17]. The global score for each treatment was defined as the mean daily sum of the erythema, edema, and scaling scores. The IS3 value (the concentration producing a global score of 3) was calculated from the dose-response curve using linear regression analysis. Six animals were used in each treatment group.

RESULTS

RAR-γ Selectivity Eleven retinoids in three structural chemotypes were synthesized and evaluated for RAR selectivity **(Fig 1)**. The structural chemotypes included allylic alcohol (compounds 1–6), hydroxymethyl naphthoate (compounds 7–10), and oxime naphthoate (compound 11) [13,14,18]. Because of the spatial orientation of the hydroxyl group, all three chemotypes can exist in two isomeric forms. Compounds 1–7 and 10 were evaluated to be racemic mixtures. Compound 7 was further resolved to either the S(−) enantiomer (compound 8) or the R(+) enantiomer (compound 9). The oxime compound 11 was tested as a 1:1 mixture of the E and Z isomers.

Retinoid selectivity for the three RARs was first determined by a transient transactivation assay in HeLa cells. All 11 retinoids were characterized to be RAR-γ selective in this assay, with RAR-γ transactivation activity greater than that of RAR-α and RAR-β **(Fig 1)**. This selectivity was examined further in a direct binding assay in which the receptor affinity was measured by binding to the recombinant receptor proteins. In general, the RAR-γ selectivity of these 11 compounds was reflected in both assays, with a minor discrepancy in compound 4. In the direct binding assay, this compound showed similar affinity for RAR-β and RAR-γ instead of a preference for RAR-γ. It should be pointed out, however, that compound 4 exhibited the lowest separation between RAR-β and RAR-γ in the transactivation assay. This compound therefore was the least selective among the 11 test retinoids.

To illustrate the receptor selectivity of the test compounds, transactivation of a representative compound (oxime compound 11) is shown in **Fig 2**. Compound 11 clearly demonstrated a preference for RAR-γ. Transactivation mediated through RAR-β

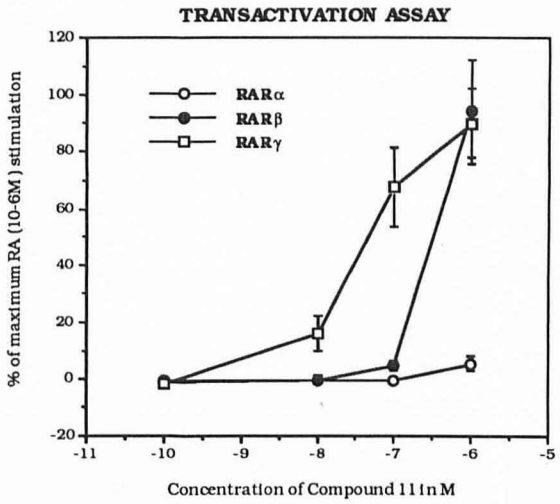
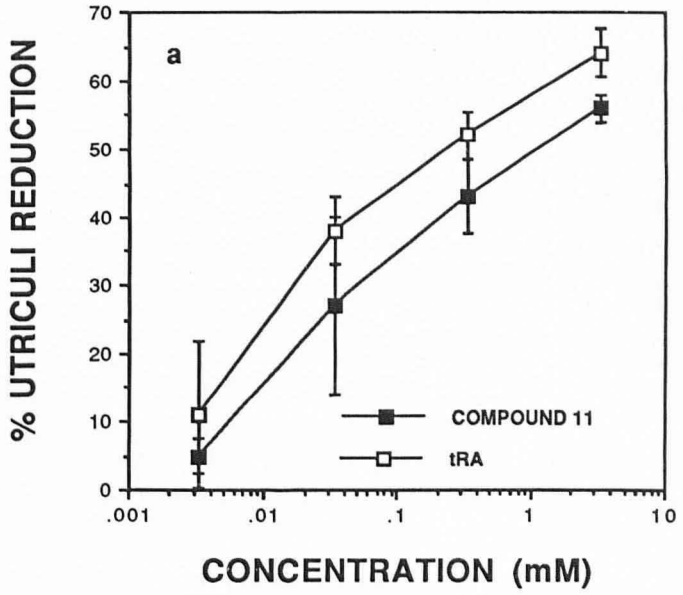


Figure 2. Illustration of RAR-γ selectivity of a representative retinoid (oxime compound 11) in the transient transactivation assay. Data represent mean ± SD from three separate experiments.



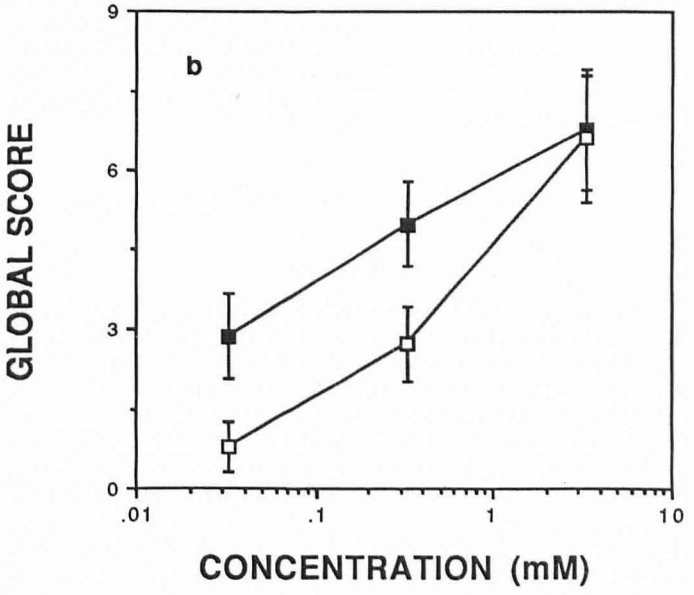


Figure 3. Demonstration of *in vivo* activity of a representative retinoid (oxime compound 11). *a*) Dose-response curve in the Rhino mouse utriculi reduction assay (n = 5). *b*) Dose-response evaluation of rabbit global irritation score after repeat doses (n = 6). *Closed squares*, compound 11; *open squares*, t-RA; *error bars*, SDs.

by this retinoid was considerably lower than for RAR-γ, and none was detected in RAR-α. The RAR-γ selectivity of this compound is also seen in its affinity. The apparent K_ds of compound 11 for RAR-α, -β, and -γ were 900, 50, and 5 nM, respectively **(Fig 1)**.

Overall, eight of the 11 compounds exhibited minimal RAR-α transactivation activity up to 10^{-6} M, and three compounds (compounds 5, 9, and 10) also showed no RAR-β–mediated activity. All of the 11 retinoids had transactivation activities in the order of RAR-γ > RAR-β > RAR-α. In contrast, the natural ligand t-RA showed similar preference for binding to the three receptors, with RAR-α transactivation activity only slightly lower than those of RAR-β and -γ **(Fig 1)**.

***In Vitro* and *In Vivo* Correlation** Each of the 11 RAR-γ–selective retinoids was tested in the Rhino mouse utriculi reduction assay and in the rabbit irritation model **(Fig 1)**. Typical dose responses in these assays are illustrated in **Fig 3**, using the oxime compound 11 as an example. At 3.3 mM, the maximum effects of potent retinoids such as t-RA and the oxime compound 11 were approximately 60% in the Rhino utriculi reduction assay, with a corresponding global irritation score of 6–7 in the rabbit. The half-maximum concentrations of these assays are therefore reported as ED_{30} and IS3, the concentration of the test retinoids effecting a 30% utriculi reduction effect or an irritation score of 3, respectively.

The compounds chosen for study demonstrated a wide range of potencies in transactivation for RAR-γ and in the *in vivo* animal models. To draw correlations, we compared the *in vivo* potencies for both utriculi reduction and skin irritation with the EC_{50} values determined in the transient transactivation assay. As seen in **Figs 4** and **5**, both *in vivo* assays correlated positively with RAR-γ transactivation activity. The correlation coefficient r was 0.94 for utriculi reduction and 0.87 for rabbit irritation (r^2, the coefficient of determination, was 0.9 and 0.8, respectively). Similar correlations also existed between apparent K_d and the *in vivo* end points, with r^2 equal to 0.7 for utriculi reduction and 0.9 for rabbit irritation (graphs not shown).

In addition to the high r^2 value, the *in vivo/in vitro* correlation is reflected in some structure-activity relations, in which a subtle change in the retinoid structure affects transactivation activity, binding affinity, and *in vivo* potency in a similar manner. The first example is the enantiomer pair compounds 8 and 9. The S(−) enantiomer compound 8 was considerably more active than the R(+) compound 9. The second example is the case of fluorination. When compounds 1 and 7 were fluorinated to compounds 2 and 10, respectively, both *in vivo* and *in vitro* activity was markedly reduced **(Fig 1)**.

It is clear from **Fig 1** that although all 11 retinoids showed preference for RAR-γ in the transactivation assay, most of these compounds also have RAR-β activity and, in some cases, RAR-α activity as well. The data in **Fig 1**, however, argue that the *in vivo* activity of these retinoids cannot be explained by their RAR-α or RAR-β activity. First of all, the eight retinoids with little or no RAR-α transactivation activity exhibited various levels of utriculi reduction or irritation activity. A notable example is the oxime compound 11, which is very potent *in vivo* but has no RAR-α activity. Furthermore, correlation plots for RAR-α and RAR-β transactivation and binding activity with the two *in vivo* assays

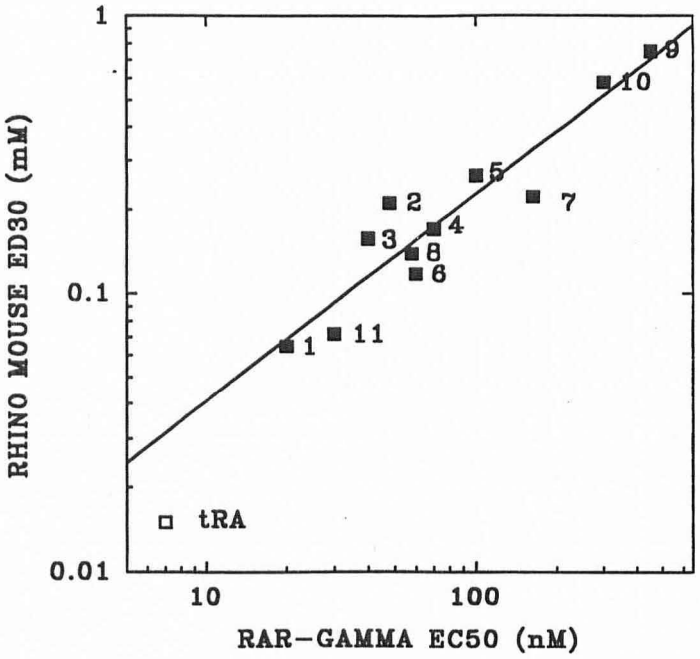


Figure 4. RAR-γ transactivation correlates with Rhino mouse utriculi reduction activity. The *in vitro/in vivo* correlations of the 11 RAR-γ–selective retinoids (*closed squares*) are made between the transactivation EC_{50} and the Rhino mouse utriculi reduction ED_{30} values. $r^2 = 0.9$. The numbers indicate the compound numbers as seen in **Fig 1**. For comparison, t-RA activity is shown (*open square*).

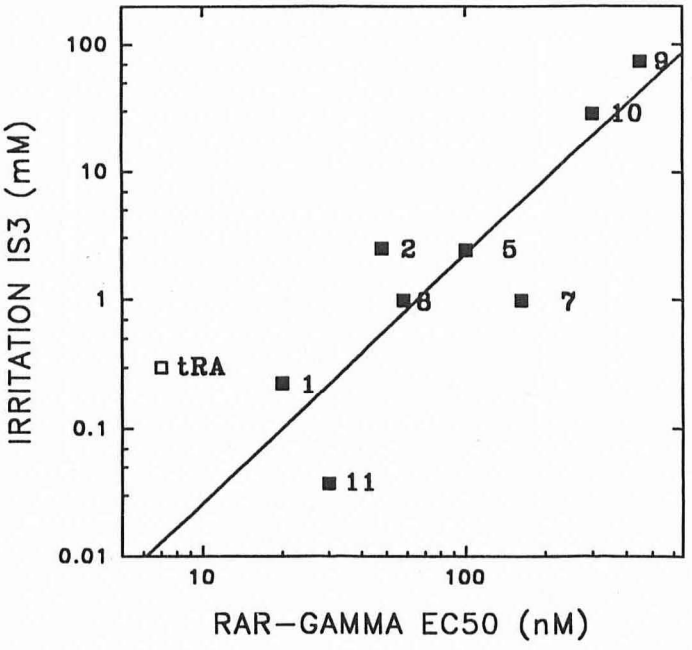


Figure 5. RAR-γ transactivation EC_{50}'s correlate with rabbit irritation IS3 values for the RAR-γ–selective retinoids (*closed squares*). $r^2 = 0.8$. The numbers indicate the compound numbers as seen in **Fig 1**. For comparison, t-RA activity is shown (*open square*).

showed no correlation, with r^2 values of 0.3 or lower (graphs not shown).

DISCUSSION

The results of this study indicate that retinoid efficacy in the Rhino mouse model and irritation in the rabbit correlate with RAR-γ–mediated transcription activation. In addition to the high r^2 values, the tight regulation of RAR-γ on *in vivo* activity is demonstrated in the structure-activity relation. In several cases, the structural differences between the test retinoids sensitively modulated both *in vitro* and *in vivo* RAR-γ activity. The most interesting examples are the two hydroxymethyl naphthoate enantiomers (compounds 8 and 9, to be published). The S(−) enantiomer compound 8 is considerably more active than the R(+) compound 9 *in vivo* and in RAR-γ activity. Another observation is that fluorination in the position ortho to the linker region decreases the efficacy. This was seen in the allylic alcohol series (compounds 1 and 2) and in the hydroxymethyl naphthoate series (compounds 7 and 10). In both pairs, the addition of a fluorine reduced both the *in vitro* and *in vivo* activity.

The nature of retinoid-induced irritation is not well understood. Because several other non-retinoid compounds such as sodium dodecylsulfate generate similar irritation in skin [22], presumably by a physicochemical mechanism, it is possible that retinoids induce irritation in a similar way. **Figures 1** and **5** show that the irritation potential of test retinoids correlates well with their *in vitro* RAR-γ activity. Because the test retinoids are structurally related, this result suggests that retinoid-induced irritation is a receptor-mediated phenomenon and is not related to the physicochemical properties of this class of compounds. The skin irritation reported with non-retinoid compounds, such as sodium dodecylsulfate [22], is most likely mediated through a different mechanism.

The functional involvement of RAR-γ in skin is consistent with its abundant expression in this organ [19]. The link between *in vivo* potency and RAR-γ but not RAR-α or RAR-β activity, as shown in this study, confirms that under physiologic conditions, RAR-γ is the predominant receptor for retinoid-mediated biologic response in the skin. However, these results still cannot exclude the possibility that RAR-α and RAR-β may also be able to initiate a dermatologic response. The three receptors may have overlapping functions in the two *in vivo* models monitored. RAR-α and RAR-β specific compounds, with little or no RAR-γ activity, are needed to address this question.

Finally, although the data presented here do not demonstrate a separation between efficacy and irritation with RAR-γ–selective retinoids, the question remains whether a RAR-γ–selective/specific compound of a different chemotype may distinguish the therapeutic benefits from the side effects. Alternatively, it may be possible to separate these effects using combinations of other receptor preferences to design a compound with a better dermatologic profile. Compounds with these characteristics are currently being evaluated.

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